A novel preparative method for the isolation of avidin and riboflavin-binding glycoprotein from chicken egg-white by the use of high-performance liquid chromatography

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A method for the rapid preparative isolation of highly purified avidin using h.p.l.c. on a powerful cation-exchanger TSK SP-5PW column has been developed. The method is based on the following properties of avidin: solubility at a high $(\text{NH}_4)_2\text{SO}_4$ concentration, stability and relatively low solubility in organic solvents, as well as the strongly cationic nature of the molecule. Riboflavin-binding glycoprotein may be isolated as by-product.

INTRODUCTION

Chicken egg-white is a rich source of nutrient-transport proteins with a specific high-affinity binding for riboflavin, thiamin, biotin and other vitamins and coenzymes [1]. Avidin, the biotin (vitamin H) carrier, has recently become an object of numerous biorecognition studies, because the avidin–biotin complex exhibits the most stable non-covalent interaction ($K_d = 10^{-15}$ M) found in Nature [2]. This property, together with an ease of modification of biotin, afforded a wide use of the avidin–biotin system in biochemistry, molecular biology, biotechnology and medicine [3]. An exceptionally extensive application of the avidin–biotin system in recent years has required a sharp increase in production of highly purified avidin. The egg-white is a unique and cheap source of this protein. Several are known for the purification of avidin. The method of Melamed & Green [4], based on chromatography on CM-cellulose, is one of the most commonly used. At the same time this technique has a few serious drawbacks. The three successive chromatography steps involved make it time- and labour-consuming, and result in a low yield. Purification of avidin on a biotinyl-Sepharose column historically appeared among the first successful applications of affinity chromatography [5]. The decisive role in the progress of affinity purification of avidin belongs to the development of chromatography on iminobiotinyl-Sepharose [6]. However, despite all the advantages of iminobiotin columns, they have serious limitations for preparative work because of their relatively low capacity, short column life and unspecific sorption. Other methods for the isolation of avidin reported include gel chromatography, metal chelate chromatography, hydrophobic chromatography and hydrophobic-interaction chromatography [7]. Most commercial avidin preparations are contaminated with other egg-white proteins and thus have diminished specific binding capacity for biotin, which makes them less efficient for some biorecognition applications. Thus a search for new convenient methods for the purification of avidin is desirable.

Previously we have developed a procedure of fractionation and h.p.l.c. purification of some egg-white glycoproteins [8]. In the present study a method was developed for a rapid preparative isolation of highly purified avidin from chicken egg-white. Accompanying riboflavin-binding glycoprotein was also isolated.

EXPERIMENTAL

Isolation of avidin from chicken egg-white

The whites of 300 eggs were separated from yolks and diluted with 0.5 vol. of water. $(\text{NH}_4)_2\text{SO}_4$ was added to 65 % saturation, and the mixture was stirred for 1 h and centrifuged at 10000 g for 20 min. The supernatant was adjusted to 100 % saturation with $(\text{NH}_4)_2\text{SO}_4$, and after 2 h the mixture was centrifuged at 10000 g for 20 min. The precipitate was dissolved in 750 ml of water and mixed with an equal volume of cooled ethanol with constant stirring for 15 min. During this procedure the temperature should not exceed -10°C . After centrifugation (at 10000 g for 15 min), the precipitate was extracted with 50 mM-sodium acetate buffer, pH 4.5. The extract was centrifuged at 10000 g for 10 min and the precipitate obtained was subjected to another extraction and centrifugation. The supernatants were combined, dialysed against water and concentrated in an Amicon cell with a PM-30 filter.

H.p.l.c.

The h.p.l.c. system used was from Waters and consisted of two pumps (model 510), injector (model U6K), gradient controller (model 721), u.v. detector (model 481 Lambda-Max) and integrator (model 730). Preparative ion-exchange chromatography was carried out on a Protein PAK SP-5PW or a Protein PAK DEAE-5PW column (21.5 mm $\times$ 150 mm) (Millipore–Waters). The pre-columns with guard gel (Beckman) were used in all separations.

Abbreviation used: RF-GPw, riboflavin-binding glycoprotein.

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Scheme 1. Isolation of avidin and RF-GPw

Avidin assay

The avidin activity was measured as described in ref. [9].

Electrophoresis

SDS/PAGE was carried out as described by Laemmli [10].

RESULTS AND DISCUSSION

The method for avidin isolation described in this paper involves two main stages: (1) isolation of the avidin-containing fraction from chicken egg-white; (2) h.p.l.c. of this fraction and isolation of avidin and RF-GPw. The sequence of isolation procedure is displayed in Scheme 1, steps I–VI.

As a preliminary step, the proteins of chicken egg-whites were fractionated by differential $(\text{NH}_4)_2\text{SO}_4$ precipitation. An initial $(\text{NH}_4)_2\text{SO}_4$ concentration of 65% saturation was chosen (Scheme 1, step I). This value was slightly less than the conventionally used 70% [6] and resulted in smaller avidin loss. Precipitation was carried out at 100% $(\text{NH}_4)_2\text{SO}_4$ saturation, affording the highest avidin recovery. SDS/PAGE of the precipitate obtained at 65–100% $(\text{NH}_4)_2\text{SO}_4$ saturation (Scheme 1, step II) allowed identification of the main remaining protein components as ovotransferrin, ovomucoid, ovalbumin, lysozyme, avidin, RF-GPw and lysozyme (Fig. 1, lane I). The next purification step used (Scheme 1, step III) was ethanol precipitation, which appeared to be very effective. SDS/PAGE has demonstrated that after precipitation with 50% (v/v) ethanol the supernatant contained virtually all ovomucoid (Fig. 1, lane II), and the extraction of the precipitate quantitatively yielded avidin

(with residual ovomucoid and lysozyme) (Fig. 1, lane III); RF-GPw was distributed among both fractions.

A few variants of the h.p.l.c. of the avidin fraction were tried. The main problem was connected with an effective separation of avidin and lysozyme, which has a high pI (11.0) and is almost co-eluted with avidin during

Fig. 1. SDS/PAGE of proteins at different stages of avidin purification

Lane I, precipitate after 65–100% $(\text{NH}_4)_2\text{SO}_4$ saturation; lane II, supernatant after ethanol precipitation; lane III, pellet extract after ethanol precipitation; lane IV, h.p.l.c.-processed pure avidin. Key: 1, ovotransferrin; 2, ovomucoid; 3, ovalbumin; 4, ovomucoid; 5, RF-GPw; 6, avidin; 7, lysozyme.

Fig. 2. H.p.l.c. of avidin fraction from 30 eggs on a protein PAK SP-5PW column

Buffer A was 1 M-acetic acid and buffer B was 1 M-acetic acid containing 1 M-ammonium acetate. The flow rate was 4 ml/min. (a) Linear gradient (curve 6 of Waters h.p.l.c. gradient controller): 0–100% buffer B in 120 min. Key: peak 1, riboflavin; peak 2, ovomucoid; peak 3, RF-GPw; peak 4, lysozyme; peak 5, avidin. (b) Convex gradient (curve 4 of Waters h.p.l.c. gradient controller): 0–100% buffer B in 120 min. Key: peak 1, riboflavin; peak 2, ovomucoid + RF-GPw; peak 3, lysozyme; peak 4, avidin.

conventional CM-cellulose chromatography. The main component of the avidin fraction, RF-GPw, has a low pI (3.8–4.1) [11] and is easily separated from other proteins. In the systems 50 mM-ammonium acetate buffer, pH 9.0,

Fig. 3. H.p.l.c. of avidin fraction from 300 eggs on a Protein PAK SP-5PW column

Conditions were as described in the legend to Fig. 2(b). Key: peak 1, lysozyme; peak 2, avidin.

with a linear gradient of $(\text{NH}_4)_2\text{CO}_3$ and 50 mM-Tris buffer, pH 9.0, with a linear gradient of NaCl an overlapping of avidin and lysozyme peaks occurred (results not shown). Complete separation of the two proteins was achieved in the system 1 M-acetic acid with a gradient up to 1 M-ammonium acetate. Two types of gradient were used: linear (Fig. 2a, curve 6 of gradient controller) and convex (Fig. 2b, curve 4 of gradient controller). In the first variant a simultaneous isolation of avidin and RF-GPw was possible. In the second variant RF-GPw was eluted immediately after the void volume and was contaminated with ovomucoid, but base-line separation of avidin and lysozyme was achieved and chromatography duration was halved. Using the latter condition, we succeeded in the separation of 700 mg of crude avidin fraction at one run (Fig. 3).

RF-GPw can be isolated independently from the supernatant after ethanol precipitation by using a Protein PAK DEAE-5PW column (Fig. 4 and Scheme 1, step VI).

As can be seen in lane IV in Fig. 1, h.p.l.c. permitted us to obtain a highly homogeneous avidin preparation, as was also confirmed by immunoelectrophoresis (results not shown). It was found to bind $14.3 \mu\text{g}$ of biotin/mg of protein. The A_{260}/A_{280} ratio was 0.68. The mean avidin yield from 300 eggs was 230 mg, or in the range of 75–85% of theoretical.

The results of this study enable us to propose a protocol of preparative isolation of avidin (Scheme 1) that differs from all conventional methods in terms of speed, yield and quality of the product. Ethanol precipitation removes the major part of ovomucoid, which is readily soluble in organic solvents [12], as well as ovotransferrin, ovalbumin and ovoinhibitors remaining in the ethanol precipitate because of denaturation. By using h.p.l.c. on a powerful cation-exchanger TSK SP-5PW column we have succeeded in the separation of avidin from RF-GPw, ovomucoid and lysozyme. The yield of avidin is 75–85% and purity approx. 99%.

Fig. 4. H.p.l.c. of RF-GPw fraction from 30 eggs on a Protein PAK DEAE-5PW column

Buffer A was 0.5 M-sodium acetate buffer, pH 5.5, and buffer B was 50 mM-sodium acetate buffer, pH 5.5, containing 0.5 M-NaCl. The flow rate was 4 ml/min. Linear gradient: 0–60% buffer B in 120 min. Key: peak 1, RFPw.

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